Novel Electro-Magnetophoretic Separation Method for the Highly Sensitive Detection of Analytes

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Supporting Information

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1 Concept of the proposed method (EMPS)

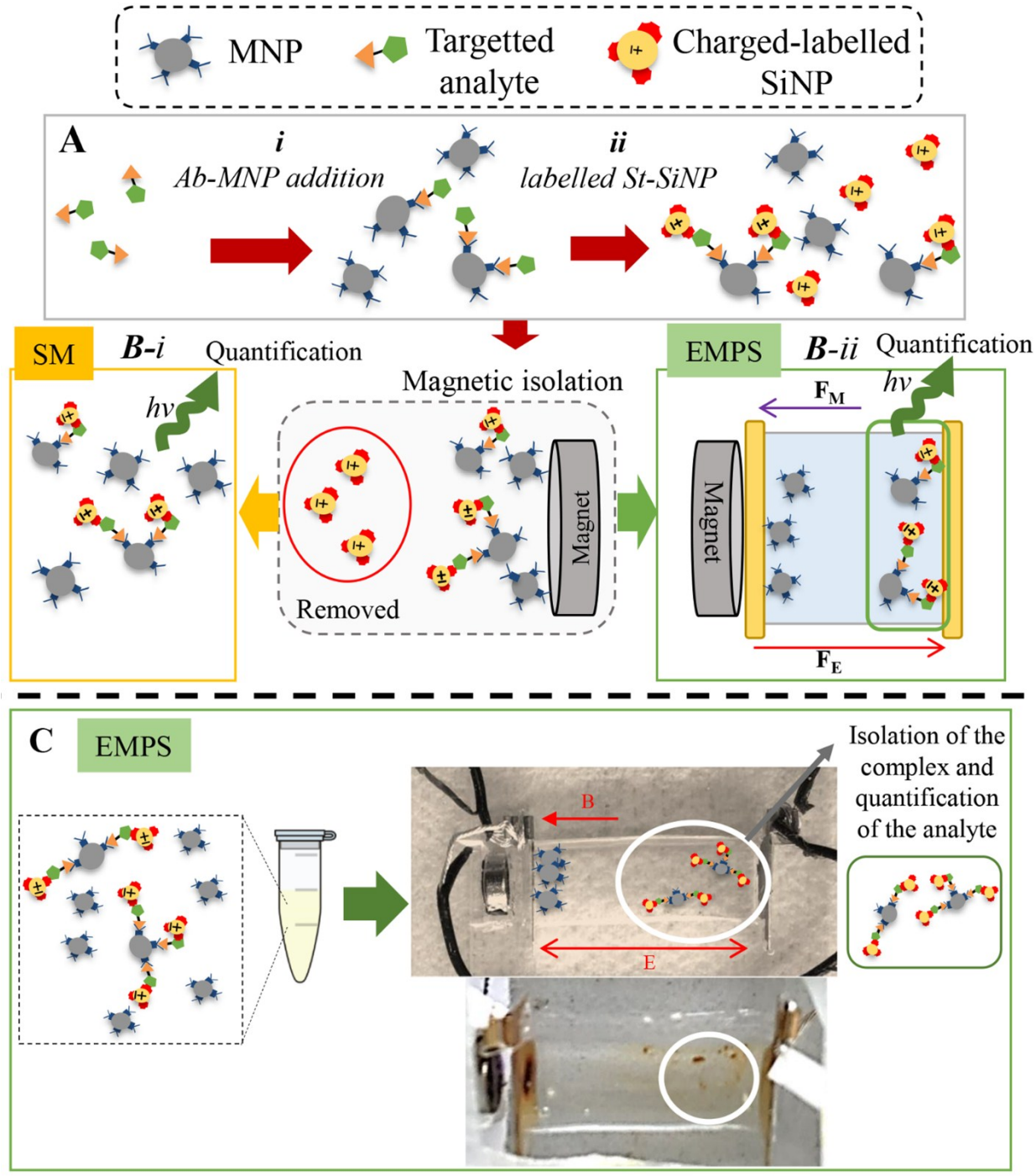

Figure S 1.1: Scheme A) shows the biological sandwich assay used for the evaluation of the presented isolation method, EMPS: i) Ab-coated MNP are added to the samples where the analyte (HRP-Bio) is present; ii) labelled streptavidin coated-SiNP are added and form the complexes with the analyte-MNP conjugate. B) Once the biological assay is completed the isolation and quantification of the analyte can be achieved by SM (B-i) or by EMPS (B-ii and C). In the first case, SM (B-i), by magnetic isolation the excess of labelled particles is removed and the quantification of the analyte is accomplished by measuring the fluorescent signal of the resulting pellet. This pellet is composed of complexes (MNP-analyte-SiNP) and of excess of MNPs. Following EMPS (B-ii and C), the pellet reached after the standard magnetic isolation is re-suspended and further purified by simultaneously applying a magnetic and electric filed. In the expressly designed device, the complexes (MNP-analyte-SiNP) can be isolated and the quantification of the analyte is calculated from the fluorescent signal emitted from the isolated fraction. The additional isolation step reduces the interferences related to the presence of excess-MNP allowing a more precise quantification of the analyte. Scheme C pictured the isolation of the complexes (toward cathode) from the excess of MNPs (toward anode and magnet) achievable by simultaneous application of magnetic and electric fields with an explanatory example of the result achieved.

2 Synthesis and characterization of nanomaterial

2.1 Synthetic procedure

- MNP

The top section of *Figure S 2.1-A* schematizes the general procedure used for the functionalization of MNP: *i*) the carboxyl groups available on the particles surface are activated by EDC/NHS (EDC only was used for Chemicell particles); The activated carboxyl groups are then coupled with the amino groups present on the HRP antibody (*ii*) and with the dye (fluorescein amine for Ab-Flu-MNP or Atto633 for Ab-Atto-MNP) (*iii*).

Synthesis of **Ab-Fluo-MNP**: 1 mg of 100 nm MNPs (chemicell) were washed twice by magnetic isolation and re-dispersion in MES 10 mM pH5. After the second wash, they were re-dispersed in MES 10 mM pH5.5 (1 mL) and shacked (500 rpm) for 10 min at RT with EDC (10 nM) and the mixture was kept shacking (500 rpm) at RT for 30 minutes. Particles were magnetically isolated and washed with PBS 10 mM pH7.4 and HRP-Ab solution was added at the concentration of 100 µg/mL. The mixture was shacked (500 rpm) for 2 h at 37°C. Ab-MNPs were magnetically isolated and 700 mL of supernatant was used to quantify the amount of Ab on the NPs surface by Bradfors assay. The pellet was further suspended in PBS 10 mM pH7.4. 10 µL of this suspension were withdraw and used for the evaluation of the antibody activity with the ABTS assay. Fluorescein amine (10 nM) was added to the suspension of Ab-MNPs and the mixture was shacked (500rpm) for 2 hours at RT. The sample was purified from the unbounded dye by 4 steps of magnetic isolation and re-dispersion in PBS 10 mM pH7.4. Ab-flu-MNP were stored in 1 mL 0.05%Tween20, 1%BSA, PBS 10 mM pH7.4 buffer at 4C.

The particles so synthesiz

ed were used for the tracking experiments due to their absorbance pack at 475 nm. The structure of such particles is schematized in *Figure S 2.1-B*.

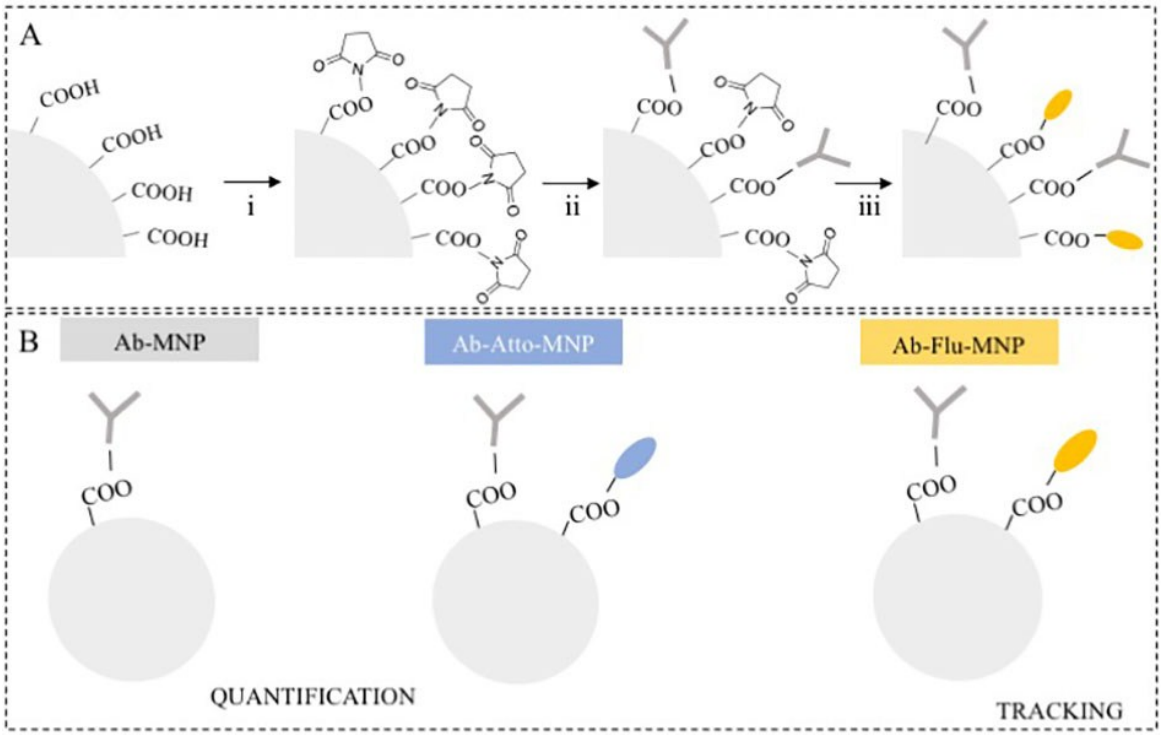

Figure S 2.1: A) Schematic representation of the general protocol used for the functionalisation of MNP: i*) the carboxyl groups available on the particles surface are activated by EDC/NHS (EDC only was used for Chemicell particles); The activated carboxyl groups are then coupled with the amino groups present on the HRP antibody (*ii*) and with the dye (fluorescein amine for Ab-Flu-MNP or Atto633 for Ab-Atto-MNP) (*iii*). B) The image shows the structure of three types of MNP synthesized and used for the experiments. Ab-MNP and Ab-Atto-MNP have been used for the quantification experiments while Ab-Flu-MNP for the tracking analysis.*

*Synthesis of **Ab-MNP**:* 1 mg of 100nm MNPs (microparticles) were washed twice by magnetic isolation and re-dispersion in MES 10 mM pH5. After the second wash, they were re-dispersed in MES 10mM pH5.5 (1mL) and shacked (500 rpm) for 10 min at RT with EDC (100 µM). NHS (150 µM) was subsequently added and the mixture was kept shacking (500 rpm) at RT for extra 20 minutes. Particles were magnetically isolated and washed with PBS 10 mM pH7.4 and HRP-Ab solution was added at the concentration of 250 µg/mL. The mixture was shacked (500 rpm) for 2 h at 37°C. Ab-MNPs were magnetically isolated and 700 mL of supernatant was used to quantify the amount of Ab on the NPs surface by Bradfors assay. The pellet was further suspended in 1% BSA solution in PBS 10 mM pH7.4. After shacking (500 rpm) at 37°C for 2h the particles were isolated and re-suspended in PBS 10 mM pH7.4. 10 µL of this suspension were withdraw and used for the evaluation of the antibody activity with the ABTS assay. Ab-MNP were stored in 1 mL 0.05% Tween20, 1% BSA, PBS 10mM pH7.4 buffer at 4C.

*Synthesis of **Ab-Atto-MNP:***

1 mg of 100 nm MNPs (microparticles) were washed twice by magnetic isolation and re-dispersion in MES 10 mM pH5. After the second wash, they were re-dispersed in MES 10 mM pH5.5 (1mL) and shacked (500 rpm) for 10 min at RT with EDC (100 µM). NHS (150 µM) was subsequently added and the mixture was kept shacking (500 rpm) at RT for extra 20 minutes. Particles were

magnetically isolated and washed with PBS 10 mM pH7.4 and HRP-Ab solution was added at the concentration of 250 µg/mL. The mixture was shacked (500 rpm) for 2 h at 37°C. Ab-MNPs were magnetically isolated and 700 mL of supernatant was used to quantify the amount of Ab on the NPs surface by Bradfors assay. The pellet was further suspended in PBS 10 mM pH7.4. 10 µL of this suspension were withdraw and used for the evaluation of the antibody activity with the ABTS assay. Atto633 (1 µM) was added to the suspension of Ab-MNPs and the mixture was shacked (500rpm) for 2 hours at RT. The sample was purified from the unbounded dye by 4 steps of magnetic isolation and re-dispersion in PBS 10 mM pH7.4. Ab-Atto-MNP were isolated and stored in 1 mL 0.05% Tween20, 1% BSA, PBS 10 mM pH7.4 buffer at 4C.

This two types of particles were used for the quantification assays and their structure is pictured in *Figure S 2.1-B*

- SiNP

SiNP were synthesized using a slightly modified version of the microemulsion quaternary method.[1,2] The procedure is schematised in *Figure S 2.2.*

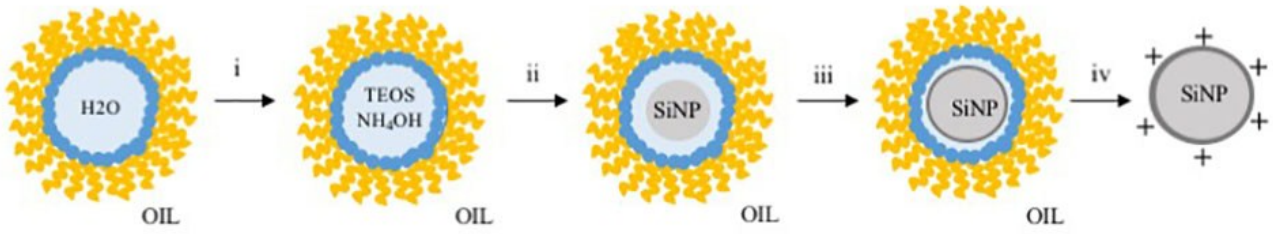

Figure S 2.2: Schematic representation of the microemulsion quaternary method used for the synthesis of SiNP: i) the microemulsion is formed combining together cyclohehexane (7.5 mL), 1-hexanol (1.133 mL), Triton® X-100 (1.894 g) and DI water (0.48 mL) in a 30 mL plastic bottle. For the synthesis of FITC-SiNP, the conjugate previously prepared FITC-APTMS is added (0.162 mL). For the synthesis of MB-SiNP, the DI water was substituted 1 mM aqueous solution of MB. 100 µL of TEOS were added followed after 30 min by 40 µL of NH4OH. ii) The reaction was stirred at RT allowing for the formation of the silica core. iii) After 24 hours, the 50 µL of TEOS were added followed after 20 min by 200 µL of APTMS for the shell formation. iv) After 24 hours ethanol was added to brake the microemulsion system. The particles were washed by centrifugation/re-dispersion cycles (x 3) in ethanol and once in water.

Dye precursor formation: In a dried glass vial, FITC (2.5 mg) was dissolved in 2 mL of 1-hexanol and APTMS (5.6 µL) was added. The reaction was allowed to proceed over 2 hours under N2 and then 0.162 mL of the mixture was collected and added into the prepared microemulsion, which preparation is described as follow.

Synthesis of ***FITC-SiNP*****:** SiNPs were formed in microemulsion prepared by combining cyclohehexane (7.5 mL), 1-hexanol (1.133 mL), Triton® X-100 (1.894 g) and DI water (0.48 mL) in a 30 mL plastic bottle under constant stirring. For the formation of the silica core, 100 µL of TEOS

were added immediately followed by 0.162 mL of the dye precursor solution. After 30 minutes, 40 µL of ammonium hydroxide (28% w/v) was added to trigger polymerization. The mixture was stirred for further 24 hours. Nanoparticle shells were synthesized by adding 50 µL of TEOS followed by 200 µL of APTMS after 20min in order to achieve positively charged particles. After 24h, the microemulsion was broken by adding 30 mL ethanol. Formed SiNPs were purified by centrifugation (14000 rpm, 10 min) and re-dispersion in ethanol (3x). After purification, the nanoparticles were stored in ethanol at 4°C.

Synthesis of ***MB-SiNP*****:** The same protocol as above was followed for the synthesis of MB-SiNP with the only difference that 1 mM solutions of methylene blue (MB) was prepared and used as the aqueous phase of the microemulsion formation.

Synthesis of ***St-G-FITC-SiNP and St-G-MB-SiNP synthesis:*** 2 mg of FITC-SiNP (or MB-SiNP) were isolated form ethanol and washed once with PBS 10 mM pH7.4. The particles pellet was then re-dispersed in a 10% solution of glutaraldehyde solution in the same buffer. The mixture was shacked (700 rpm) at RT for 75 minutes. The sample was then centrifuged and the so achieved pellet was washed twice with PBS 10 mM pH7.4. The particles suspended in this buffer were reacted with Streptavidin (100 µg/mL) and the mixture was incubated at 37°C overnight under shacking (500rpm). The day after particles were isolated by centrifugation (1000 rpm 10 min). 700 mL of supernatant was collected to quantify the amount of Streptavidin remain on the surface by Bradford assay. Particles were stored in 0.5% Tween20, 1%BSA in PBS 10 mM pH7.4 at 4°C.

The functionalization procedure used for the synthesis of St-G-FITC-SiNP and St-G-MB-SiNP is pictured in *Figure S 2.3-A.* The final structure of the SiNP used for tracking and quantification analysis are shown in *Figure S 2.3-B.*

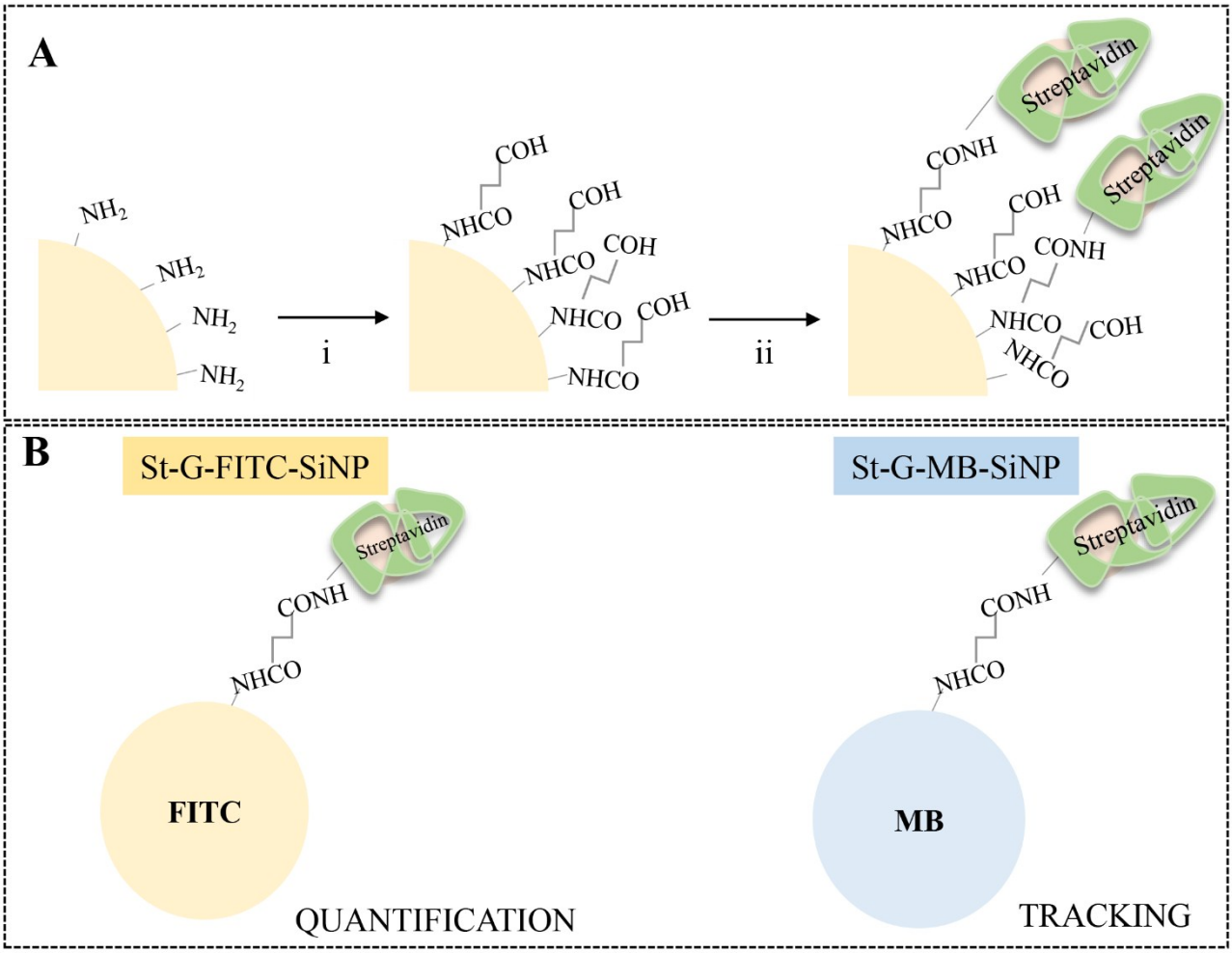

Figure S 2.3: A) Schematic representation of the protocol followed for the general functionalization of SiNP: i) taking advantage of the amino groups present, the surface of the particles has been coated with Glutaraldehyde in order to expose aldehyde groups; ii) this more reactive groups have been then use as anchor for the attachment of Streptavidin. B) the structure of the two types of SiNP used is presented. St-G-FITC-SiNP were used for quantification assays while St-G-MB-SiNP were used for tracking experiments.

2.2 Characterization

All types of particles were characterized at each step of the functionalization procedure by DLS, TEM and spectrophotometrically.

- MNP

Figure S 2.4 shows the structure (A) and TEM image (C) of Ab-Flu-MNP. The UV-Vis spectrum of the bare MNP (*Figure S 2.4-B*; grey line) and of Ab-Flu-MNP (orange line) were recorded. Whereas the MNP have a broad absorbance peak at *c.a.* 350 nm, Ab-Flu-MNP have a narrow peak at 476 nm. Hydrodynamic diameter and zeta potential were measured by DLS for each step of functionalization (D).

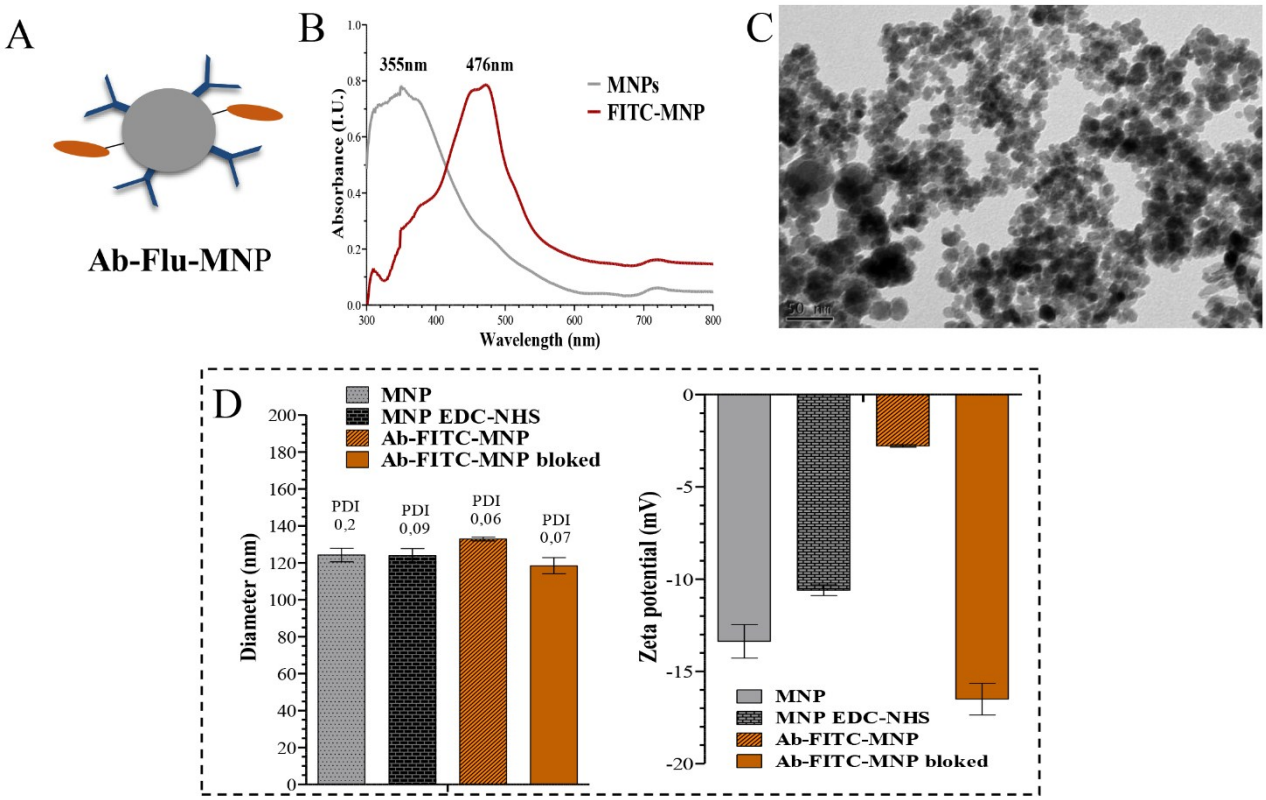

Figure S 2.4: A) Schematic structure of Ab-Flu-MNP. B) UV-Vis spectrum of the particles before functionalization (grey line- MNPs) and after functionalization with Ab and fluorescein amine (orange line- Ab-Flu-MNP). Whereas plane MNP have a broad absorbance peak at c.a. 350 nm, Ab-Flu-MNP absorb at 476 nm. C) TEM image of Ab-Flu-MNP. D) Size and zeta potential measured by DLS analysis of the particles at each step of functionalization for 10 µg/mL of particle in filtered DI water.

Table S 2.1 shows the DLS value measured for the MNP at each step of functionalization. As noticeable, while the diameter varies negligibly at the different steps (ranging from a minimum diameter of 118.43 up to a maximum of 132.93 nm for the Ab-Flu functionalized particles), the surface charge becomes almost neutral (-2.78 ± 0.07) after functionalization and turn back to negative after incubation with the blocking buffer. This could be due to the different hydrophilicity acquired by the particles once most of the surface is covered by protein (antibodies and BSA). This altered hydrophobicity, changing the thickness of the double layer surrounding the particles, lead to the alteration of the NP's hydrodynamic diameter, which is the one measured by DLS.

Table S 2.1: Size and zeta potential measured by DLS analysis of the particles at each step of functionalization for 10 µg/mL of particle in filtered DI water. Average value (n = 3) ± SD.

	Size (d-nm)		PDI		ZP (mV)	
	Average	**±SD**	**Average**	**±SD**	**Average**	**±SD**
MNP	124.23	3.69	0.20	0.01	-13.37	0.91
MNP EDC-NHS	123.93	3.84	0.09	0.01	-10.60	0.29
Ab-Flu-MNP	132.93	1.05	0.06	0.00	-2.78	0.07
Ab-Flu-MNP BSA1%	118.43	4.36	0.07	0.01	-16.50	0.86

Figure S 2.5 the structure of Ab-MNP (i), the corresponding TEM image recorded (ii) and the size and zeta potential measured by DLS at each step of functionalization (iii).

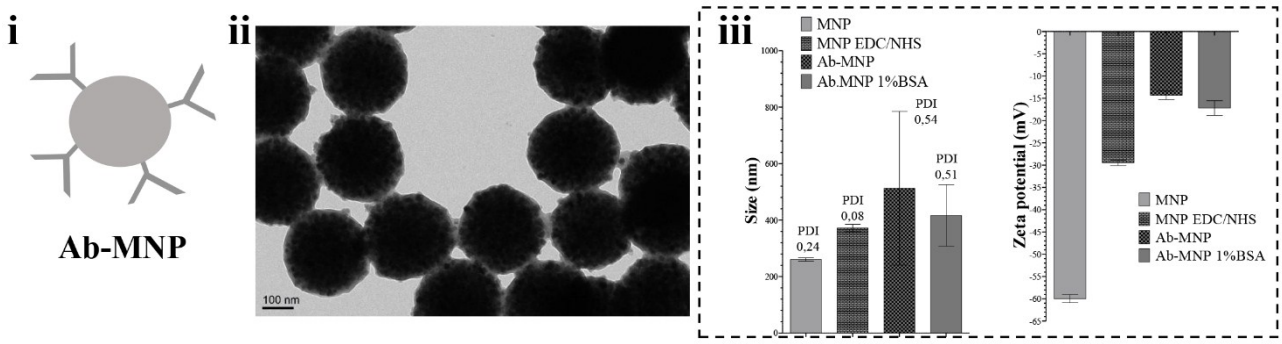

Figure S 2.5: Structure (i) and TEM (ii) of Ab-MNP; iii) DLS data related to size and zeta potential of the particles at each step of functionalization.

Table S 2.2 shows DLS and TEM data measured for each step of functionalization for Ab-MNP. The hydrodynamic diameter of the particles increased during the functionalization, starting from 260.30 ± 5.96 nm for the bare MNP particles to 512.73 ± 271.87 nm for the Ab-MNP. Even in this case particles slightly shrinks after incubation with the blocking buffer (416.47 ± 109.1 nm). The real diameter measured by TEM instead increased only lightly (from 203.26 ± 14.5 nm for the MNP up to 252.40 ± 16.21 nm for the final particles). The variation of the surface charge clearly indicates the success of the functionalization: ZP became more positive during the functionalization steps starting from the very negative bare MNP (-59.97 ± 0.92 mV) and the final Ab-MNP (-17.20 ± 1.65 mV).

Table S 2.2: DLS data measured for the particles at each step of functionalization measured for 10 µg/mL of particle in filtered DI water. Data are shown as average value (n = 3) ± SD. The diameter measured by analyzing TEM image with ImageJ software are reported as average value of c.a. 50 NP ± SD.

	Size (nm)		**PDI**		**ZP (mV)**		**Size (nm) TEM**	
	Average	**± SD**	**Average**	**± SD**	**Average**	**± SD**	**Average**	**± SD**
MNP	260.30	5.96	0.24	0.02	-59.97	0.92	203.26	14.5
MNP EDC-NHS	371.80	13.00	0.08	0.02	-29.70	0.29		
Ab-MNP	512.73	271.87	0.54	0.06	-14.33	0.94		
Ab-MNP BSA1%	416.47	109.1	0.51	0.04	-17.20	1.65	252.40	16.21

Figure S 2.6 shows the structure (i) and TEM image (iii) of Ab-Atto-MNP. The spectrophotometric properties of this particles were evaluated by scanning a suspension of 50 µg/mL Ab-Atto-MNP in DI water. UV-Vis spectrum (*Figure S 2.6-ii,* grey line) and fluorescent spectrum (*Figure S 2.6-ii,* blue line) indicate that such particles have an absorbance peak at 630 nm and a fluorescence peak at 655 nm. Size and zeta potential were measured by DLS at each functionalization step (*Figure S 2.6-iv)*.

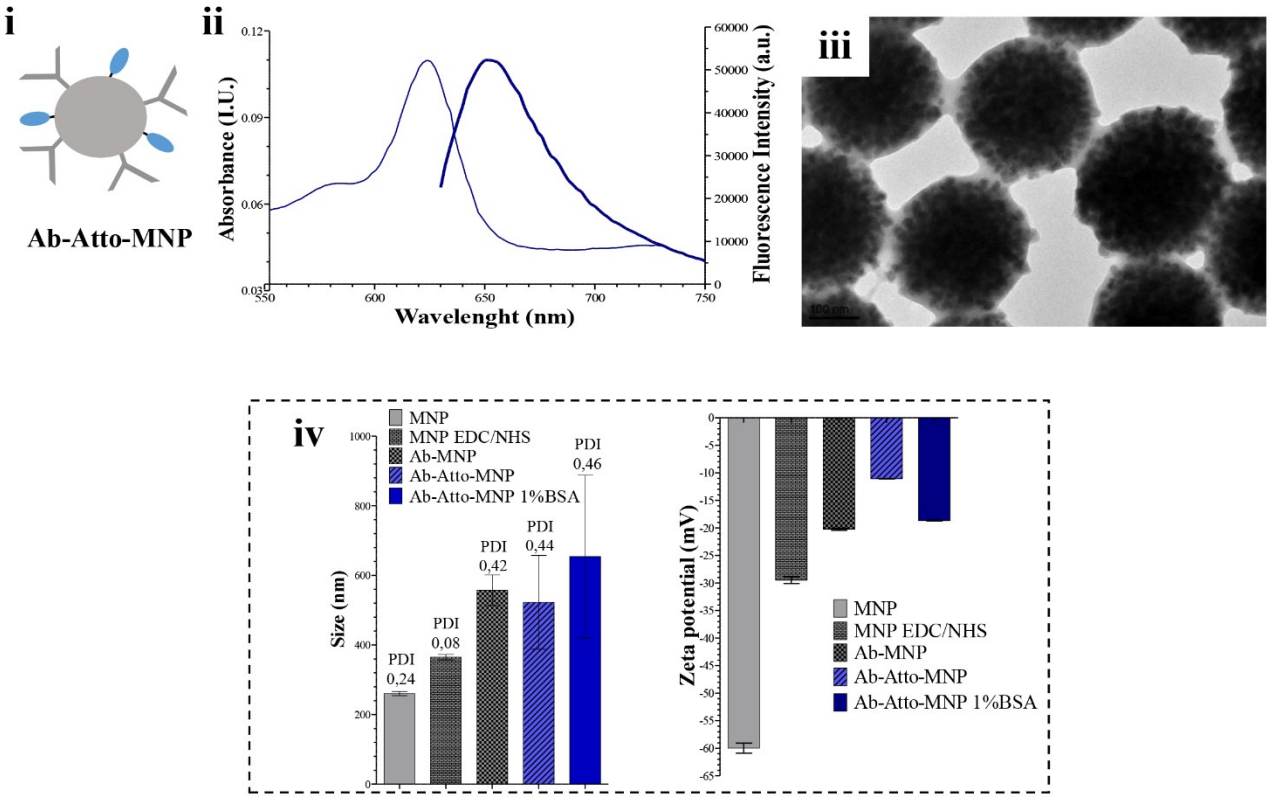

Figure S 2.6: i) Schematic structure of Ab-Atto-MNP. ii) UV-Vis spectrum (grey line) and fluorescent spectrum (blue line) for the 50 µg/mL in Di water. Ab-Atto-MNP absorb at 630 nm and emit at 655 nm. iii) TEM image of Ab-Flu-MNP. iv) Size and zeta potential measured by DLS analysis of the particles at each step of functionalization for 10 µg/mL of particle in filtered DI water.

Table S 2.3 shows DLS data measured for each step of functionalization for Ab-Atto-MNP. The diameter of the particles was evaluated by analyzing TEM image as well for the bare and final particles. The hydrodynamic diameter of the particles increased during the functionalization, starting from 260.30 ± 5.96 nm for the bare MNP particles to 653.70 ± 233.90 nm for the final Ab-Atto-MNP. The real diameter measured by TEM confirmed the success of the functionalization since an increase of the size was observed (from 203.26 ± 14.5 nm for the MNP up to 274.94 ± 11.77 nm for the final particles). Even for these particles the zeta potential became more positive with the functionalization (from -59.97 ± 0.92 mV- MNP- until -18.67 ± 0.09 mV -Ab-Atto-MNP-).

Table S 2.3: DLS data measured for the particles at each step of functionalization measured for 10 µg/mL of particle in filtered DI water. Data are shown as average value (n = 3) ± SD. The diameter measured by analyzing TEM image with ImageJ software are reported as average value of c.a. 50 NP ± SD.

	Size (nm)		PDI		ZP (mV)		Size (nm) TEM	
	Average	**± SD**	**Average**	**± SD**	**Average**	**± SD**	**Average**	**± SD**
MNP	260.30	5.96	0.24	0.02	-59.97	0.92	203.26	14.5
MNP EDC-NHS	371.80	13.00	0.08	0.02	-29.70	0.29		
Ab-MNP	557.10	44.02	0.42	0.06	-20.23	0.19		
Ab-Atto-MNP	522.47	134.81	0.44	0.08	-11.07	0.05		
Ab-Atto-MNP 1% BSA	653.70	233.90	0.46	0.04	-18.67	0.09	274.94	11.77

- SiNP

Figure S 2.7 shows the schematic structure of St-G-MN-SiNP (A). B) The UV-Vis properties of the particles were characterized by measuring the absorbance scan for two samples at 100 and 500 µg/mL of particles concentration in DI water. Due to the loaded MB in the silica core, such particles present two absorbance peaks, at 560 and 690 nm respectively. At high concentration (500 µg/mL) the peak at 690 nm is hidden by the large intensity of the first peak at 560 nm. However, at lower concentration (100 µg/mL) the intensity of the two peak is more similar and even the peak at 690 nm becomes suitable for the identification of these particles. TEM images for St-G-MB-SiNP indicate that the particles have a homogeneous spherical shape and they are well monodispersed (*Figure S 2.7-C)*. Hydrodynamic diameter and zeta potential were measured by DLS for 10 µg/mL of particles in filtered DI water (*Figure S 2.7-D)*.

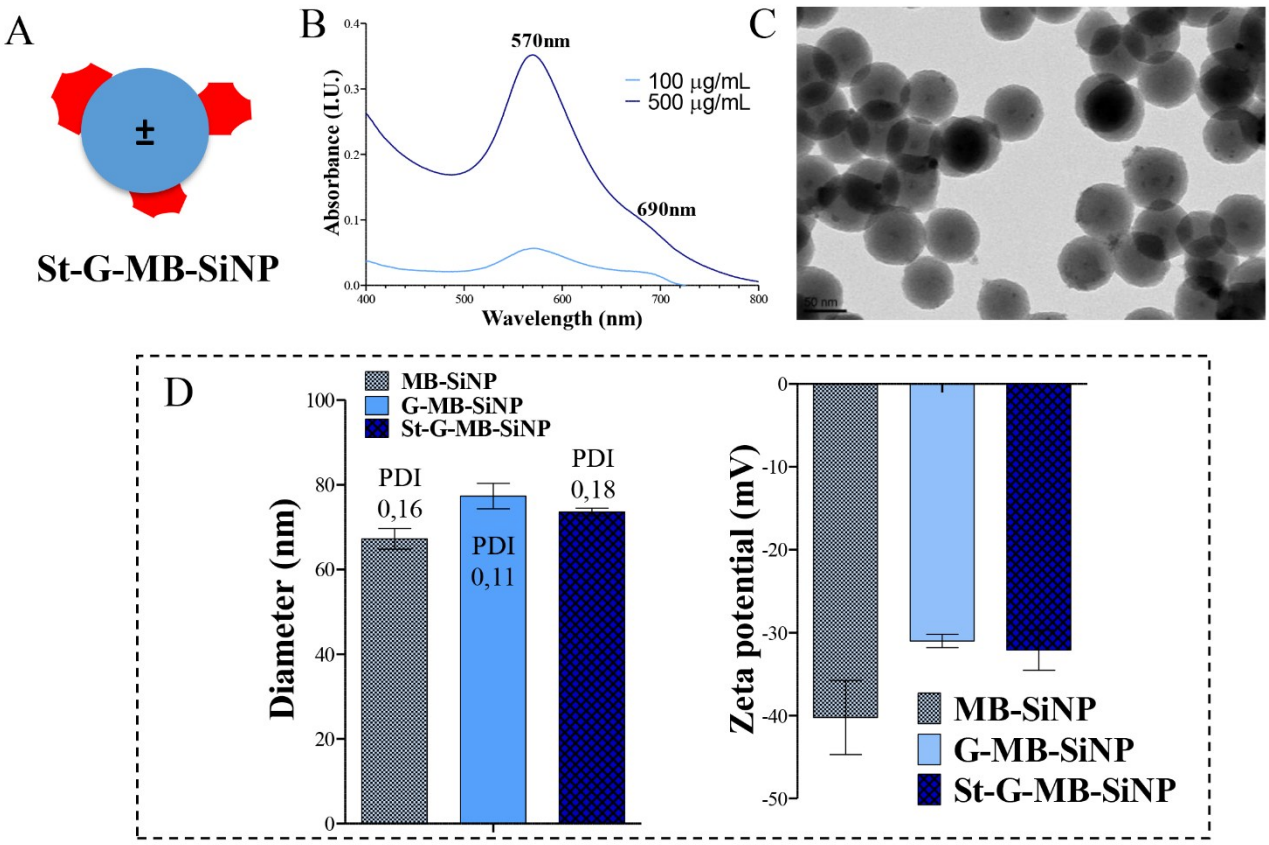

Figure S 2.7: A) Schematic structure of St-G-MB-SiNP. B) UV-Vis spectrum of the particles at different concentrations: while at high concentration (500 µg/mL) the particles have basically a sharp absorbance peak at 570 nm (dark blue line), at lower concertation (100 µg/mL) beside the peak at 570 nm a second peak acquire relevance at 690 nm (light blue line). C) TEM image of St-G-MN-SiNP. D) Size and zeta potential measured by DLS analysis of the particles at each step of functionalization for 10 µg/mL of particle in filtered DI water.

Table S 2.4 shows the DLS values measured for 10 µg/mL of particles dispersed in filtered DI water. During the functionalization the diameter increased slightly (from 67.24 ± 2.44 to 73.57 ± 0.93) and the ZP became less negative (from -40.23 ± 4.46 to -32.10 ± 2.44) indicating that the functionalization was successfully achieved.

Table S 2.4: DLS data measured for the particles at each step of functionalization measured for 10 µg/mL of particle in filtered DI water. Data are shown as average value (n = 3) ± SD.

	Size (d-nm)		PDI		ZP (mV)	
	Average	±SD	Average	±SD	Average	±SD
MB-SiNP	67.24	2.44	0.16	0.01	-40.23	4.46
G-MB-SiNP	77.33	3.01	0.11	0.01	-31.00	0.80
St-G-MB-SiNP	73.57	0.93	0.18	0.01	-32.10	2.44

Figure S 2.8 shows the schematic structure of St-G-FITC-SiNP (i). ii) The spectrophotometric properties of this particles were evaluated by scanning a suspension of 100 µg/mL St-G-FITC-SiNP in DI water. The UV-Vis spectrum (light orange line) and fluorescent spectrum (dark orange line) indicate that due to the presence of FITC in the silica matrix such particles have an absorbance peak at 490 nm and a fluorescence peak at 525 nm. TEM images for St-GFITC-SiNP indicate that the particles have a spherical shape but the samples appear polydispersity in terms of size (*Figure S 2.8-C)*. Hydrodynamic diameter and zeta potential were measured by DLS for 10 µg/mL of particles in filtered DI water (*Figure S 2.8-D)*.

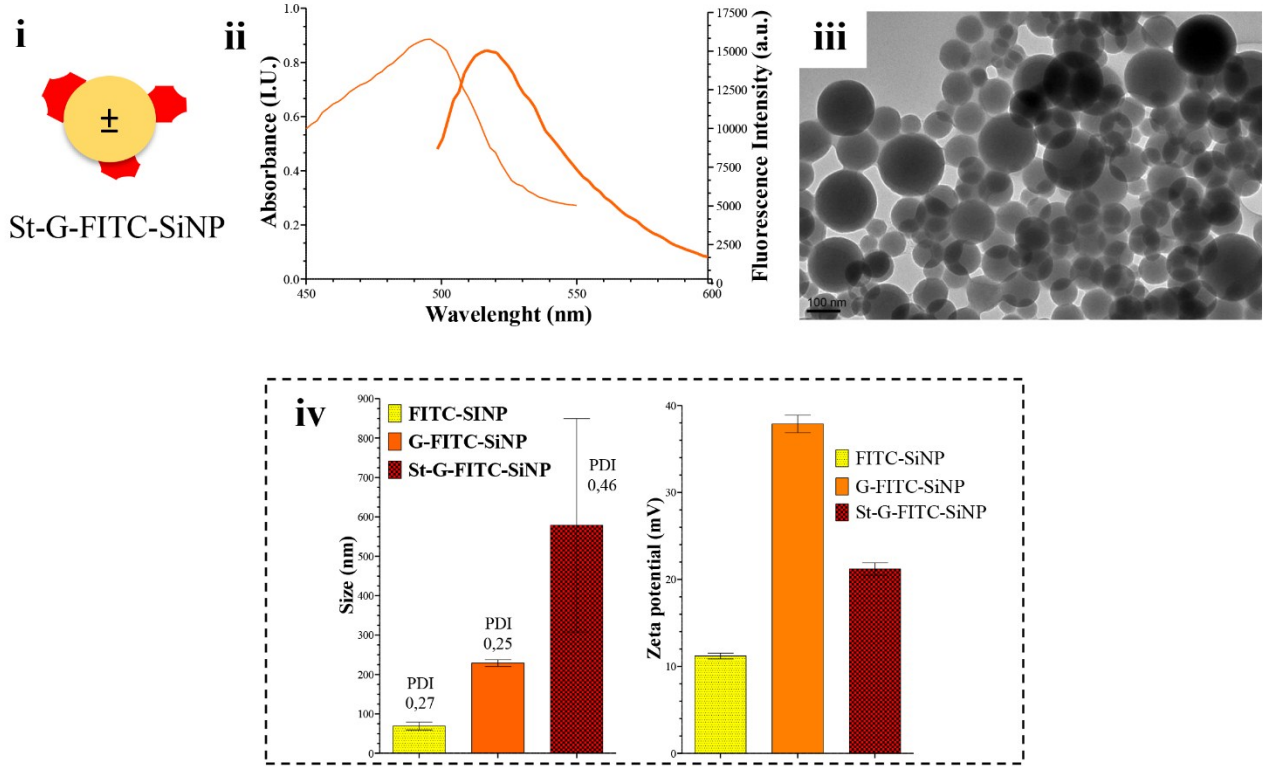

Figure S 2.8: A) Schematic structure of St-G-MB-SiNP. B) UV-Vis spectrum of the particles at different concentrations: while at high concentration (500 µg/mL) the particles have basically a sharp absorbance peak at 570 nm (dark blue line), at lower concertation (100 µg/mL) beside the peak at 570 nm a second peak acquire relevance at 690 nm (light blue line). C) TEM image of St-G-MN-SiNP. D) Size and zeta potential measured by DLS analysis of the particles at each step of functionalization for 10 µg/mL of particle in filtered DI water.

Table S 2.5 shows DLS values measured at each functionalization step. The diameter increased remarkably (from 69.23 ± 9.98 to 578.67 ± 270.66 for FITC-SiNP and St-G-FITC-SiNP respectively) and the ZP became more positive (from -40.23 ± 4.46 to -32.10 ± 2.44) indicating that the functionalization was successfully achieved.

Table S 2.5: DLS data measured for the particles at each step of functionalization measured for 10 µg/mL of particle in filtered DI water. Data are shown as average value (n = 3) ± SD.

	Size (d-nm)		PDI		ZP (mV)	
	Average	±SD	Average	±SD	Average	±SD
FITC-SiNP	69.23	9.98	0.27	0.05	11.20	0.33
G-FITC-SiNP	228.90	8.61	0.25	0.02	37.87	1.01
St-G-FITC-SiNP	578.67	270.66	0.46	0.06	21.10	0.71

2.3 Protocols for quantification procedures

Coomassie (Bradford) protein assay:

The collected supernatant was further centrifuged in order to isolate at the bottom eventually present particles (14000rpm, 10 min). 150 µL/ 5 µL mixed with 150 µL/250 µL of Coomassie (Bradford) Protein Assay Kit reagent for respectively low and high concentration of protein expected. The plate is incubated for 10 minutes at RT and the absorbance is read at 595 nm with Multiskan GO (Thermo Fisher). For each measurement blank and reference sample containing the amount of protein used for the experiment were evaluated as well.

ABTS assay:

10 µg/mL of Ab-MNP were incubated at 37°C with 100 µg/mL of HRP-Bio (500 rpm shacking). PBS 10 mM pH 7.4 was used as buffer. After 2 hours, the particles were magnetically isolated. After re-dispersion in the same volume of buffer, 5 µL of both MNP-suspension and supernatant were added to the wells of a 96-well microplate. In the meantime, the following solutions were prepared: A) 100 mM potassium phosphate buffer pH 5.5 was prepared adjusting the pH of 100 mM potassium phosphate monobasic solution by adding 100 mM potassium phosphate dibasic solution; B) ABTS 50 nM in DI water; C) 30% (w/w) solution of H_2O_2 was diluted reaching the concentration of 0.025% (w/w). These solutions were mixed together in the ration of 22:7:1 (A:B:C). The mixture was shacked for 15 minutes at RT in dark and 300 µL were added to the each well of the 96-well microplate in which 5 µL of each sample was previously added. The absorbance was read every 5 minutes for 30 minutes at 436 nm. Un-reacted particles and the buffer were used as blank for the MNP-suspension samples and the supernatant respectively. HRP-Bio at the know concentration used during the assay was used as reference for the 100% activity of the HRP. The amount of active HRP present on the Ab-MNP was calculated from the absorbance signal measured.[3,4]

Biotin-FITC assay:

100 µL of St-SiNP suspension and 100 µL of biotinylated FITC were mixed in each well of a 96-well microplate reaching as final concentration 10 µg/mL for the St-SiNP and 25 nM of biotin-FITC. The microplate was shaken (150 rpm) at RT for 30 minutes. PBS 10 mM pH 7.4 was used as buffer. Fluorescent signal was measured using 480-525 nm as excitation and emission wavelength respectively. The signal detected indicates the amount of FITC-Bio which did not interact with the streptavidin on the NP surface. Indeed, the recognition between streptavidin and biotin quenches the FITC's fluorescence. Lower is therefore the signal detected and higher is the active streptavidin present on the NP surface. Plane SiNP were used as blank and PBS buffer was used as 100% intensity value. The reduction of the fluorescent signal was used to calculate the amount of active side of streptavidin on the surface.[5]

Buffers preparation:

Phosphate buffers at different pH were prepared mixing 0.2 M sodium phosphate dibasic and 0.2 M sodium phosphate monobasic, diluting it at the concentration of 100, 10, 5 and 1 mM and adjusting the pH to 6 and 7.4 using 5 M NaOH and 5 M of HCl. 10 mM MES buffers at different pHs were prepared starting from a stock solution of 0.1 M of MES. After dilution to 10 mM with DI water and NaOH (1 M) or HCl (1M) were added until the desired pH was reached. Buffers: **B-1:** 1.67 mM Na_2SO_4, 1 mM NaOH pH10.8; **B-2:** 1 mM Na Citrate, 2 mM Na_2SO_4 pH7.1; **B-3:** 0.03 mM phosphate buffer pH 6.5; **B-4:** 0.03 mM phosphate buffer pH 5.5; **B-5:** 1 mM Na Citrate, 2 mM Na_2SO_4 pH4.

3 Evaluation of environmental parameters

Considering that not only particles feature but even environmental parameters determine the electrophoretic their behavior, we experimentally investigated some parameters which we can control in order to achieve the desired result. All measurements were accomplished suspending 10 µg of each type of particles in 1 mL of each buffer.

3.1 Buffers analysis

The solvent in which the nanomaterials are suspended has a key role since it determines the surface charge of the particles and the conductivity of the suspension. For this reason, the zeta potential (ζ, mV) and conductivity (C, mS/cm) of both MNP and SiNP were evaluated by DLS in different buffers.

In particular, the particles were tested in PBS pH 6.5 at different concentrations (200, 100, 10, 5 and 1 mM) and in 10 mM MES at different pHs (3-8 pH).

In the case of MNPs, ζ decreases with the increase of the pH changing from – 4.35 ± 1.84 mV at pH3 to – 19.40 ± 1.95 mV at pH5, becoming almost neutral at pH7 (– 0.02 ± 0.24 mV). The C in this case is usually low; the maximum conductivity is reached at pH8 (1.47 ± 0.08 mS/cm) at which correspond a relatively negative ζ (-15.77 ± 0.90 mV) but it almost zero mS/cm was measured at pH7 (*Figure S 3.1 i and iii)*. At difference concentration of the buffer, ζ of MNP fluctuates between -7.69 ± 0.20 mV (10 mM) and -12.57 ± 4.73 mV (100 mM) but it becomes neutral at 200 mM. As consequence, C drops from 12.30 ± 0.33 mS/cm (10 mM) to zero at concentration of 200 mM (*Figure S 3.1 ii and iv)*.

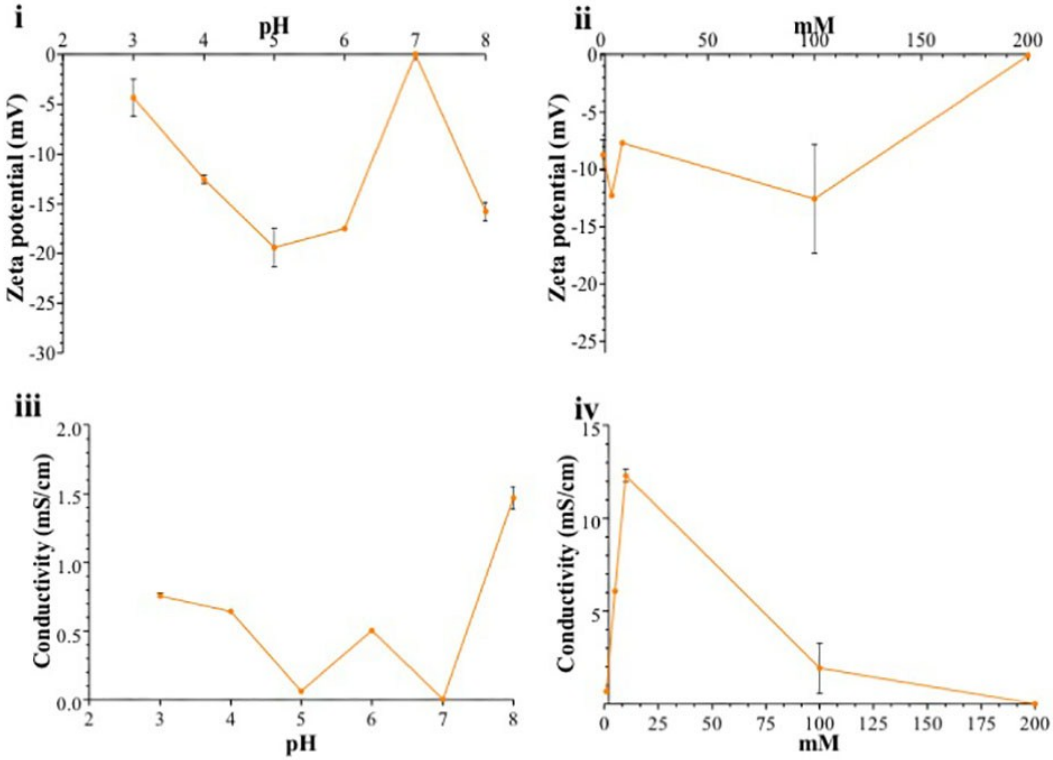

Figure S 3.1: Relationship between ζ and C of MNP suspension and differ conditions. i) ζ of MNP in PBS pH6.5 at 200, 100, 10 ,5 and 1 mM concentrations. ii) ζ of the MNP in 10 mM MES at different pH ranging from 3 until 8. iii) C of MNP in PBS pH6.5 at 200, 100, 10 ,5 and 1 mM concentrations. iv) C of the MNP in 10 mM MES at different pH ranging from 3 until 8.

Different situation was measured for SiNP. While ζ is slightly positive in acidic conditions (pH3, 5.59 ± 0.76 mV) it becomes negative in basic environments (pH8, -12.87 ± 0.12 mV). At pH5 ζ for SiNP become neutral which led to a C almost zero while the highest value of conductivity was measured for SiNP at pH 8 (0.6 ± 0.15 mS/cm) (*Figure S 3.2 i and iii)*. Analyzing the data achieved for SiNP suspended in PBS buffers at different concentrations we observed that C increase with the

increase of the ζ, suggesting that in such case, the ion concentration of the buffers determines the conductivity of the suspensions and not the surface of charge of the particles. In particular, ζ increased from -23.97 ± 1.09 mV to -12.07 ± 0.82 mV for respectively 10 mM and 200 mM of the concentration tested which correspond to the lower (0.91 ± 0.03 mS/cm) and the highest (14.43 ± 0.81 mS/cm) conductivity (*Figure S 3.2 ii and iv)*.

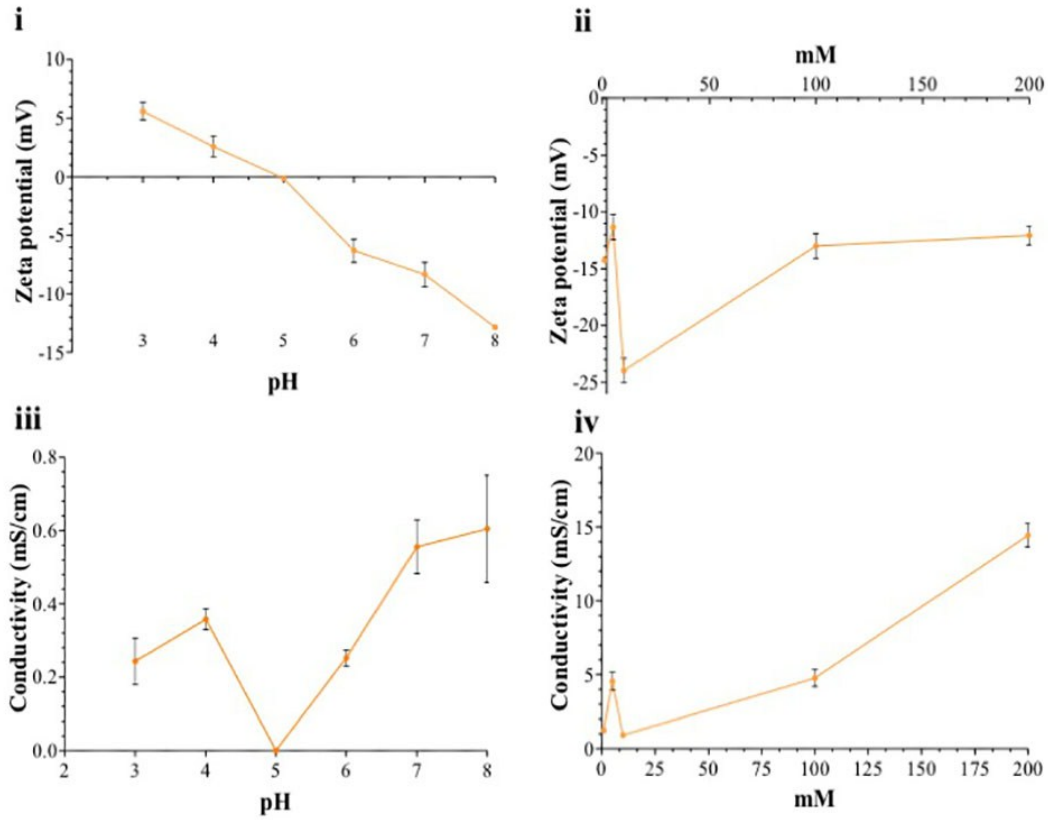

Figure S 3.2: Relationship between ζ and C of SiNP suspension and differ conditions. i) ζ of SiNP in PBS pH6.5 at 200, 100, 10 ,5 and 1 mM concentrations. ii) ζ of the SiNP in 10 mM MES at different pH ranging from 3 until 8. iii) C of SiNP in PBS pH6.5 at 200, 100, 10 ,5 and 1 mM concentrations. iv) C of the SiNP in 10 mM MES at different pH ranging from 3 until 8

Furthermore, the similar analysis was accomplished using more complex buffers. ζ and C values measured for each particle type in these different buffers were compared. The buffer which gave the better combination of results between for MNP and SiNP was selected for the following tracking and quantification analysis.

Figure S 3.3 shows ζ and C values measured for Ab-Flu-MNP (*A* and *C* respectively*)* and for St-G-MB-SiNP (*B* and *D* respectively*)*. In order to achieve a good separation, buffer **B-1 with 0.001% of** agarose was selected as solvent for the tracking analysis. Indeed, using this solvent Ab-Flu-MNP have a ζ neutral while St-G-MB-SiNP have a negative surface charge, almost -30 mV. The conductivity for both particles in such buffer is around 1 mS/cm. This combination of buffer and nanomaterial was chosen for the tracking analysis.

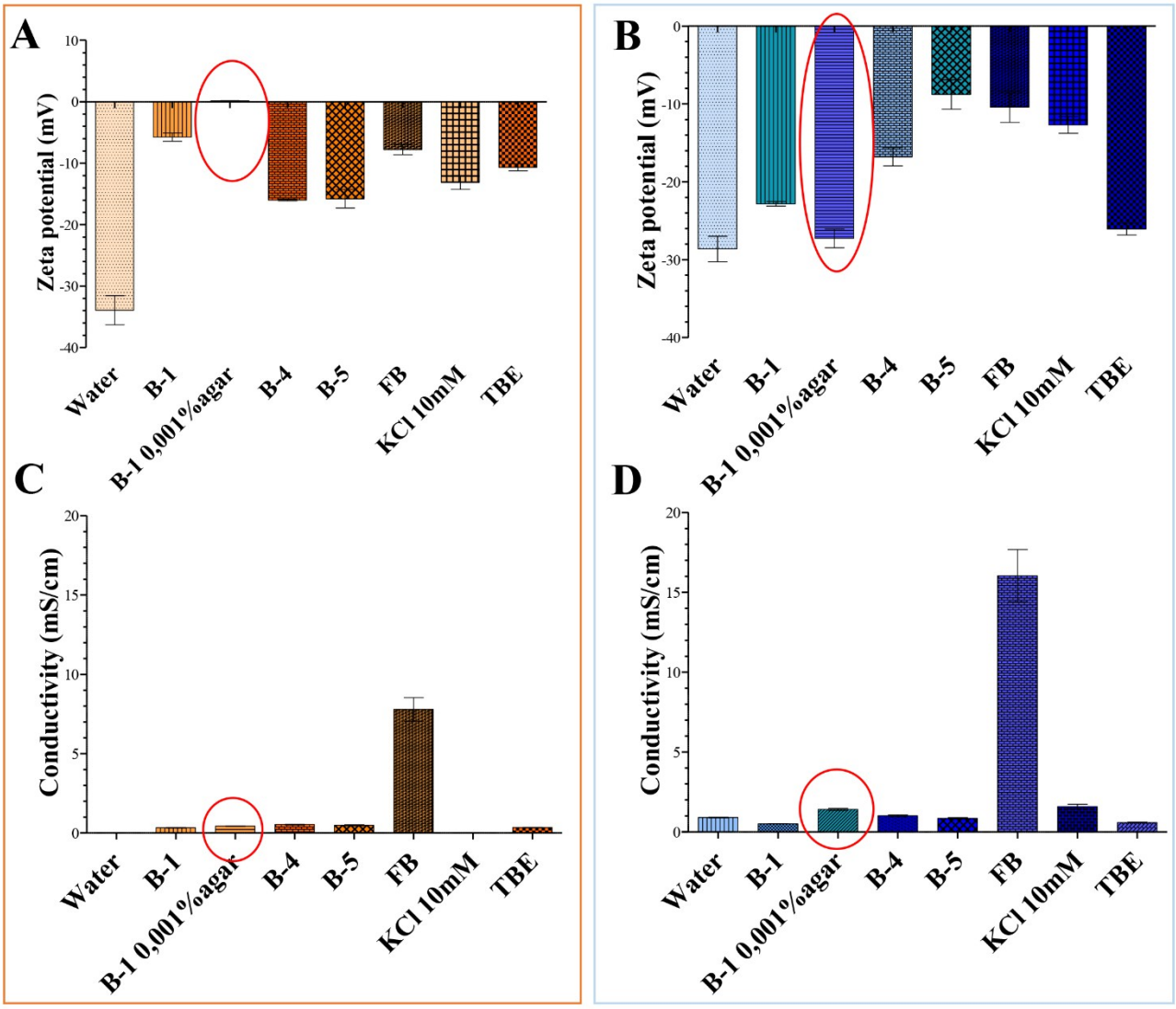

Figure S 3.3: DLS data achieved for Ab-Flu-MNP measuring ζ (A) and C (C) in different buffers. DLS data achieved for St-G-MB-SiNP measuring ζ (B) and C (D) in different buffers. B-1 0,001% of agarose was chosen as the suitable buffer for tracking analysis with these particles.

Figure S 3.4 shows ζ and C values measured for Ab-Atto-MNP (blue bars) and Ab-MNP (grey bars) (*A* and *C* respectively*)* and for St-G-FITC-SiNP (*B* and *D* respectively*)*. In order to achieve a good separation, buffer 10 m **MES** pH4 was selected as solvent for the quantification assay. Indeed, while MNP (Ab-Atto-MNP and Ab-MNP) have a ζ close to the neutrality (5 and 0 mV respectively), in MES pH4 St-G-FITC-SiNP are highly positively charged, almost 20 mV. The conductivity for both particles in such buffer is around 1 mS/cm. This combination of buffer and nanomaterial was chosen for the quantification assays.

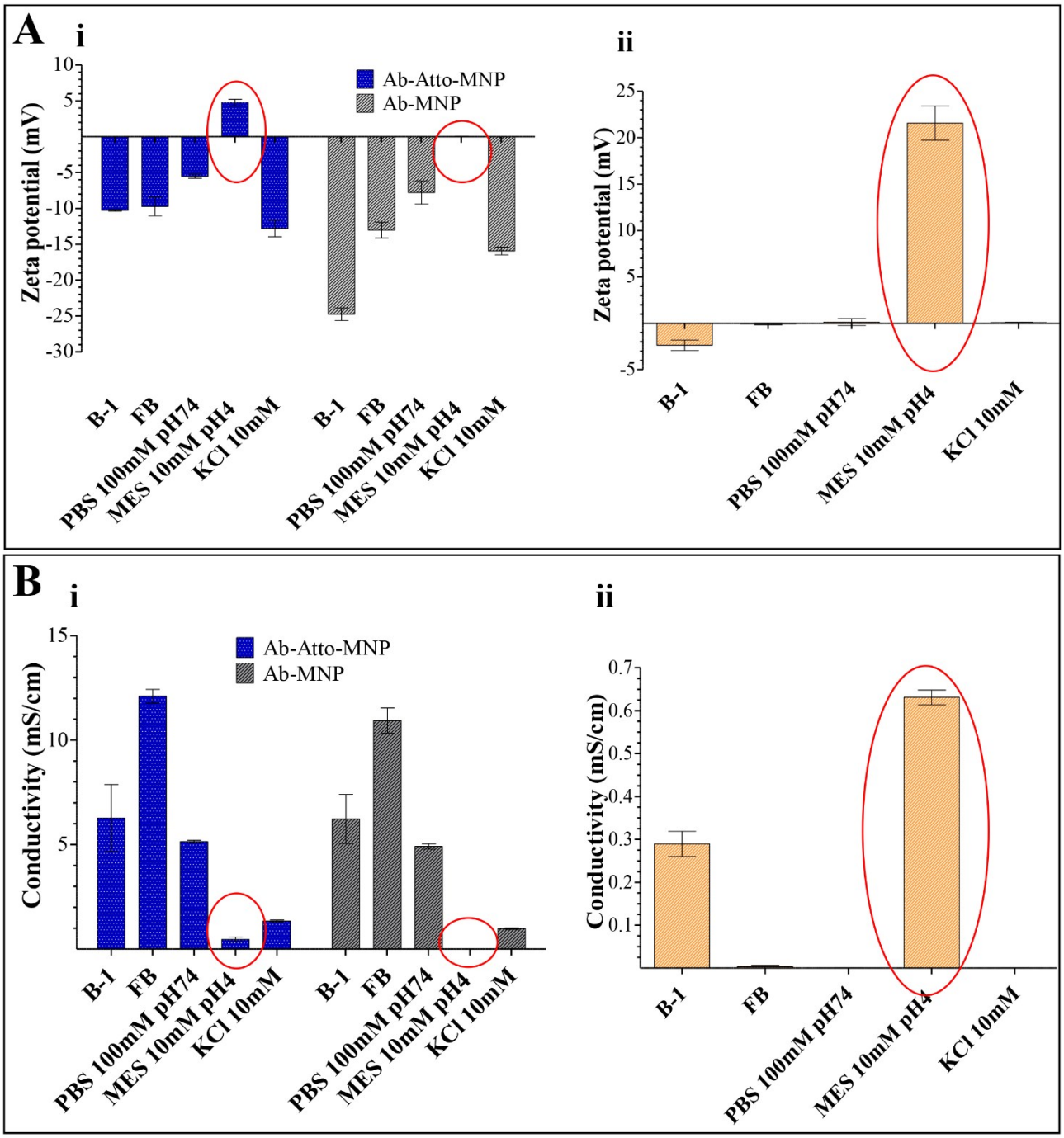

Figure S 3.4: DLS data achieved for Ab-Atto-MNP(blue bars) and Ab-MNP (grey bars) measuring ζ (A) and C (C) in different buffers. DLS data achieved for St-G-FITC-SiNP measuring ζ (B) and C (D) in different buffers. 10 mM MES pH4 was chosen as the suitable buffer for quantification assays with these particles.

4 Tracking analysis

Tracking experiments were accomplished using a PDMS chamber with a magnet on one side (B 500 mT). The electric field (1 V, 1 mA) was generated by placing two gold coated cupper electrodes at the two sides. As schematized in the top part of *Figure S 4.1* the chamber was designed in order to be divided in three parts corresponding to a well of a 384-well microplate. The yellow part is the one closer to the magnet and to the cathode; the grey part corresponds to the middle section of the chamber while the blue part is the one closer to the anode. In this way, the movement of the particle in the chamber was evaluated by measuring the absorbance at 475 nm (Ab-Flu-MNP) and 700 nm (St-G-MB-SiNP) selecting the corresponding three well in the software of the microplate reader (Multiskan GO, Thermo Fisher). From the signal measured from each of the three parts of the chamber, the % incremental curves were calculated and plotted in a graph as a function of time.

General experimental conditions: the pellet isolated after the first isolation step is further suspended in buffer suitable for electrophoresis (1.67 mM Na_2SO_4 1 mM NaOH pH10.8 (B-1) with 0,001% agarose). 10 µL of this suspension is added to the PDMS chamber (100 µL Vol_{tot}). The movement of the particles in presence of magnetic field (B = 500 mT) and electric field (E = 1 V, 1 mA) is tracked by measuring the absorbance at 475 nm and 700 nm for respectively Ab-Flu-MNP and St-G-MB-SiNP respectively in correspondence of the three sections in which the chamber is divided (yellow, grey and blue sections). The absorbance was measured before application of the electric field (t0) and subsequently every minute for 20 min.

Figure S 4.1-A shows the results achieved using St-G-MB-SiNP only: as observed the signal decreases with the time in the yellow and gray sections while increases rapidly after 8 minutes at the blue section indicating that St-G-MB-SiNP accumulate as expected at the anode.

Figure S 4.1-B shows the results achieved using Ab-Flu-MNP only: as observed in this case the signal decreases with the time in the blue and gray sections while increases suddenly after the first minutes at the yellow section indicating that magnetic particles accumulate towards the magnet and cathode.

In *Figure S 4.1-C and D* are shows explanatory results obtained adding to the chamber 10 µL of sample achieved after the accomplishment of the biological assay and after the first isolation step. In *Figure S 4.1-C* (detection of 1.44 µM of HRP-Bio) is noticeable a rapid increase of the signal related to Ab-Flu-MNP towards the magnet/cathode. This indicates that the excess of MNP accumulated on this side of the chamber and the fact that the signal decreases suddenly to zero can be due to the fact that once the particles adhere at the electrode they are anymore detectable. Interestingly, an increase of both signals related to both MNP and SiNP can be observed in the blue part of the chamber. The fact that two types of particles moved with the same trend toward the anode indicate that they are likely moving as single entity *i.e.* the complexes. Indeed, in a situation where no complexes were

expected (*Figure S 4.1-D*) the signal increases for St-G-MB-SiNP (blue line) while decreases for Ab-Flu-MNP (orange line) closer to the anode, following a different trend, which indicate that they moved independently not as complex.

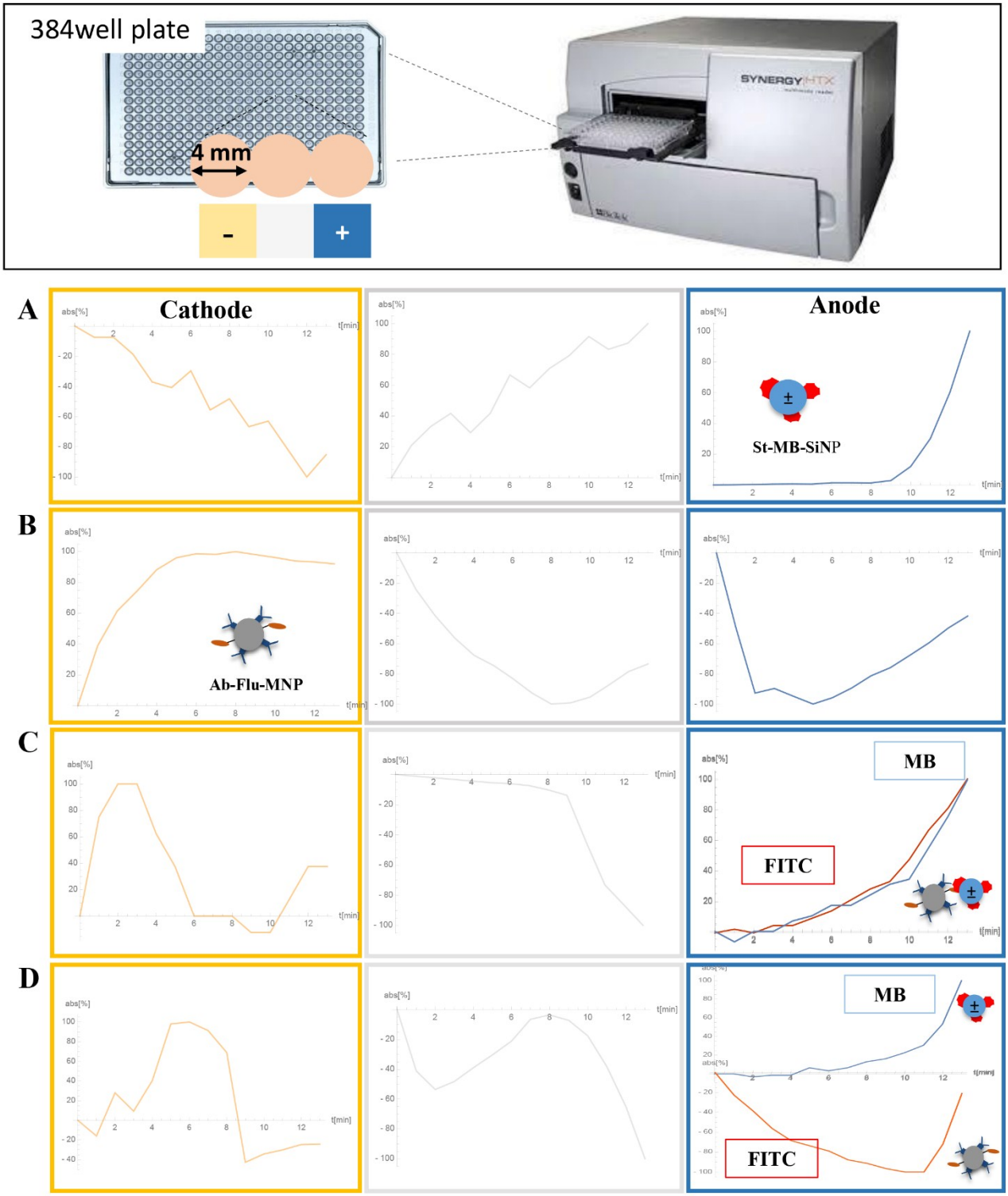

Figure S 4.1:A) tracking of St-G-MB-SiNP in the chamber during the experiment. B) Tracking of Ab-Flu-MNP in the chamber during the experiment. C) Tracking of the isolated sample after the sandwich assay for the detection of 4,55 µM HRP-Bio and after the first isolation step. D) Tracking analysis of a mixture of Ab-Flu-MNP and St-G-MB-SiNP.

In the middle part of *Figure S 4.2* some two examples of the results achieved during the tracking experiments. It was possible to notice by naked eye the separation occurred during the assay: a brownish fraction can be observed on the left side of the chamber, closer to the anode and on the other side, toward the magnet, a brown deposit adherent to the cathode was clearly distinguished. 5 µL in proximity of both fraction were withdraw and washed twice by centrifugation/re-dispersion cycles in order to remove the agarose fibers present in the buffer used for the tracking experiments. The samples collected from the isolated fractions were spotted on a TEM grid and analyzed. On the left, TEM analysis of the isolated fractions on the cathode, closer to the magnet. As noticeable, this fraction was mainly composed of MNP, while both particles were distinguished in the fraction isolated closer to the anode (*Figure S 4.2*, right images). The presence of both St-G-MB-SiNP and Ab-Flu-MNP on one side while only Ab-Flu-MNP were present on the other side confirmed the results achieved by absorbance measurement and indicated that the removal of the excess of MNP and the isolation of the analyte can be accomplished by mean of the herein proposed method.

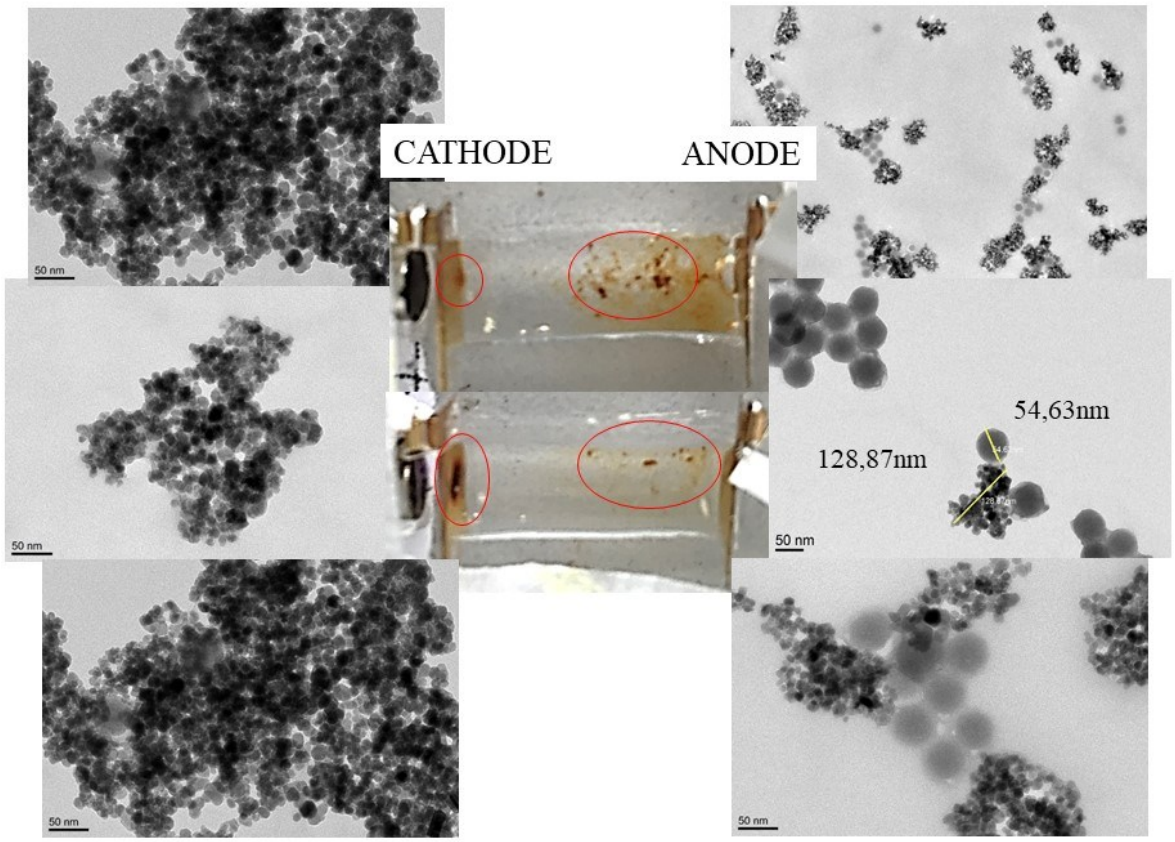

Figure S 4.2: In the middle, some examples of the separation achievable after the assay based on simultaneous application of magnetic and electric fields. Two fractions can be distinguished in the chamber: the one closer to the magnet/cathode is composed almost by MNP (TEM images on the left) while on the other side both particles are clearly distinguished by TEM analysis (images on the right). This confirmed that EMPS effectively allows to remove the excess of MNP isolating selectively analyte-MNP conjugates.

5 Evaluation of EMPS

5.1 Selectivity

The selectivity of the biological sandwich immunoassay was tested using Protein A in the assay instead of the specific analyte (HRP-Bio). The assay was accomplished three time for each isolation method. To a solution of 100 µM of Protein A, Ab-Atto-MNP (µg/mL of particles corresponding to 200 nM of Ab) were added (500 µL final volume). The mixture was incubated at 37°C under shaking 500 rpm. PBS 10 mM pH 7.4 was used as buffer. After 2 hours, MNP were isolated by applying a magnet removing 490 µL of supernatant. The pellet was re-dispersed in blocking buffer (1% BSA solution in PBS 10 mM pH 7.4) and incubated for 30 minutes. After magnetic isolation of the pellet, HRP-Ab-MNP were re-suspended in fresh buffer and St-G-FITC-SiNP were added (µg/mL of particles corresponding to 200-500 nM of active sites of streptavidin). The mixture was incubated at 37°C under shacking (500 rpm). After 30 minutes, applying a magnet HRP-Ab-Atto-MNP were isolated from the excess of St-G-FITC-SiNP removing 490 µL of supernatant. The pellet was washed once in the MES 10 mM pH 4 in order to remove all the excess of SiNP without disturbing the pellet and it was finally suspended in 10 µL of MES 10 mM pH 4.

SM: *5* µL of the isolated fraction were spotted on a clean microscope slide. After the drop dried off, the remaining 5 µL were added over it. The dried spot was analysed by ChemiDOC MP (BioRAD) using the FITC filter.

EMPS: this 10 µL were further purified in the designed PDMS chamber (50 µL Vol_{tot}). The pellet is further suspended in buffer suitable for electrophoresis (B-1 for Ab-Flu-MNP and MES 10 mM pH 4 for Ab-Atto-MNP). This suspension is added to the PDMS chamber. The sample is further purified by applying simultaneously a magnetic field (B = 270 mT) and an electric field (500 mV, 1 mA). After 20 minutes, 15 µL were carefully withdraw simultaneously at each electrode. The isolated fractions were concentrated by centrifugation (800 rpm, 4 minutes) and removal of 5 µL of the supernatant. The remaining 10 µL were vortexed and spotted (5 µL twice) on a glass microscope slide.

Figure S 5.1 shows the results obtained after quantification of the SiNP isolated. In both cases, the signal detected was negligible as the corresponding quantification of the protein (6.38 µM and 1.42 for respectively SM and EMPS).

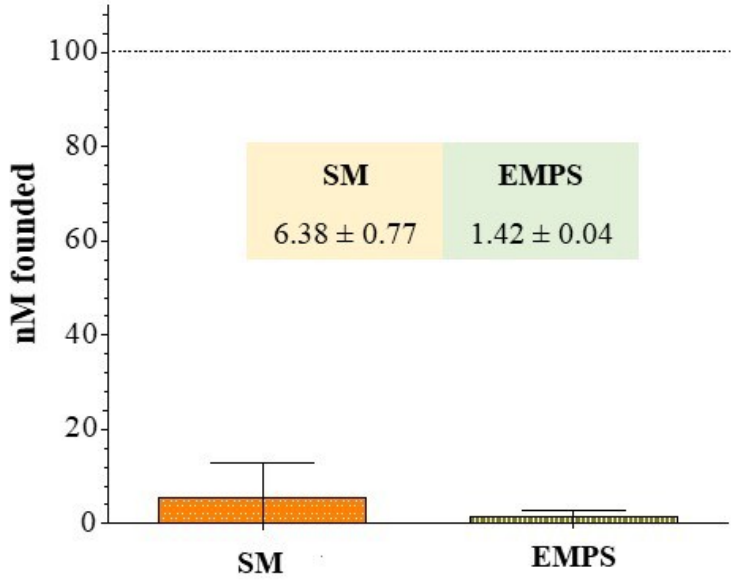

Figure S 5.1: Evaluation of the selectivity of the biological sandwich immunoassay used for this study. Results suggested that the assay is reasonably selective for the targeted analyte, HRP-Bio. Data are shown as average value (n = 3), ± SD.

5.2 Reproducibility

The reproducibility of the method was evaluated reproducing the experiment with the same amount of analyte several times in independent experiments (n = 6). *Figure S 5.2* shows the results achieved for measuring 10 (*i*) and 0.1 nM (*ii*) of HRP-Bio. Result indicate that using EMPS as isolation method, the analyte can be isolated with good precision (12.3 nM and 0.12 nM are the values found using 10 and 0.1 nM of analyte respectively) and with good reproducibility between different experiments considering the small standard deviation calculated considering 6 independent experiments.

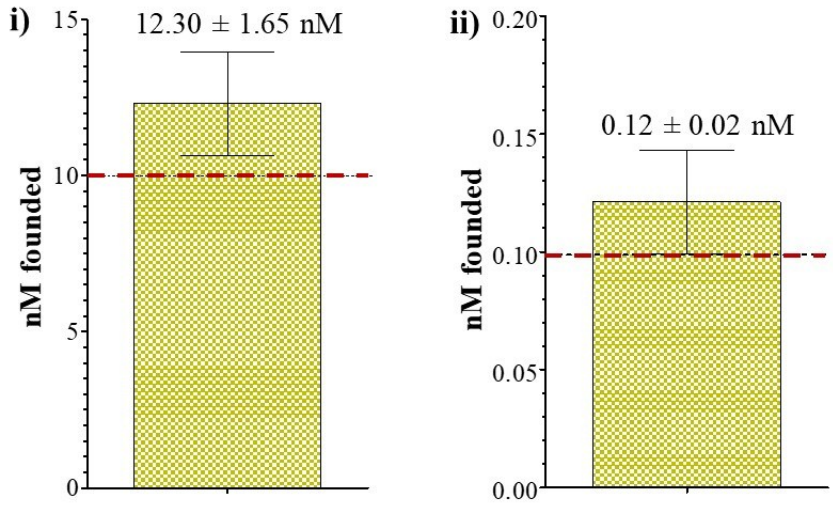

Figure S 5.2: The values achieved in detecting 10 and 0.1 nM of HRP-Bio by EMPS method indicates good reproducibility and sensitivity of the method. Data are shown as average value (n = 6), ± SD.

5.3 Robustness

The two methods (SM and EMPS) were further compared in complex environment, which better mimic human samples. Therefore, the biological assay was accomplished using DMEM and FBS instead of PBS as buffer for the HRP-Bio suspension. To the solution in DMEM/FBS of 0, 0.01, 1 and 100 mM HRP-Bio Ab-Atto-MNP were added reaching 200 nM as final concentration of Ab. After incubation, the MNP were magnetically isolated and the pellet suspended in PBS. The protocol proceeded as mentioned above. Data are reported in *Table S4.1* and are plotted in *Figure S 5.3*. As noticeable, whereas no much difference in terms of sensibility was noticed using DMEM as buffer between the two methods (*Figure S 5.3-i;* 15.88 nM and 5.93 nM were the values calculated respectively by SM and EMPDS) better results were noticed in the case of FBS. In such case indeed, the sensitivity of EMPS was better in detecting 1 nM of the analyte compared with SM (*Figure S 5.3-ii*; 1.24 mM was detected by EMPS, 4.36 nM by SM) and furthermore, by EMPS the value achieved in detecting 0.01 nM was less than 1 order of magnitude higher than the expected (0.78 nM) compared to the value obtained with SM which was almost 10-folds higher (8.26 nM).

Table S 5.1: Quantification of HRP-Bio initially dispersed in complex medium (DMEM and FBS) by means of SM and EMPS.

	DMEM		**FBS**	
nM	**SM**	**EMPS**	**SM**	**EMPS**
0	15.36	0.60	5.91	8.77
0,01	14.23	4.85	8.26	0.78
1	15.88	5.93	4.36	1.24
100	94.23	68.84	109.59	55.23

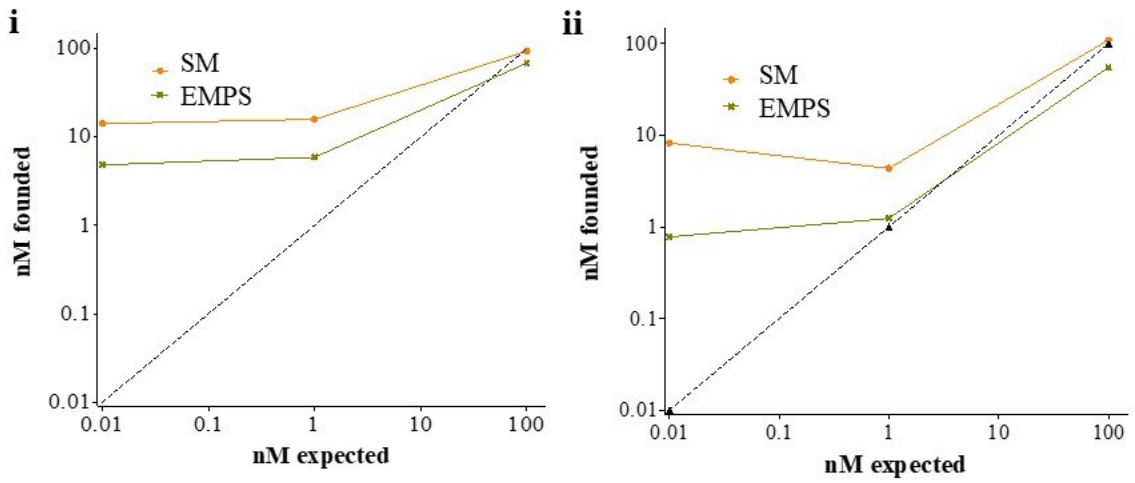

Figure S 5.3: The values achieved in detecting HRP-Bio in DMEM (i) and FBS (ii) with both SM and EMPS are plotted against the expected values.

5.4 Sensitivity

To clear the air regarding the efficiency of the proposed isolation and detection approach here in proposed, we accomplished other experiments in which the labelled-MNP were substituted with un-labeled particles, Ab-MNP. To a PBS solution of HRP-Bio at different concentrations ranging from 0 up to 100 nM, Ab-MNP were added reaching a final concentration of 200 nM of Ab in the samples. The isolation and detection procedure was accomplished as usual. The experiment was accomplished with 6 independent experiments.

Table S 5.2 shows the results achieved with the two methods. An overestimation of the analyte in solution was observed even using un-labeled MNP at low concentrations. For the expected concentrations of 0.01 and 0.1 nM the found values were indeed 4.78 and 0.58 nM with SM. Exploiting instead the further isolation, the values found were closer to the one expected (0.10 nM and 0.20 respectively for 0.01 and 0.1 nM).

Table S 4.2: Quantification of HRP-Bio by means of the two compared Methods.

	SM	EMPS
	Average	**Average**
0	4.03	0.3
0.001	1.24	0.04
0.01	2.58	0.10
0.1	0.58	0.12
1	4.98	1.31
10	30.23	15.29

100	96.04	92.86

In *Figure S 5.4,* in which the values found are plotted against the expected one. It is easily to see that SM leads to an overestimation of the analyte if present al low concentration and removing the excess of MNP the detection is more accurate even in such situations. This is translated into the improvement of 10-folds the LOD, 1 nM is the LOD for SM while 0.1 nM for LOD) which is confirmed by values of the slop calculated for EMPS which indicates an improved sensitivity compared to SM.

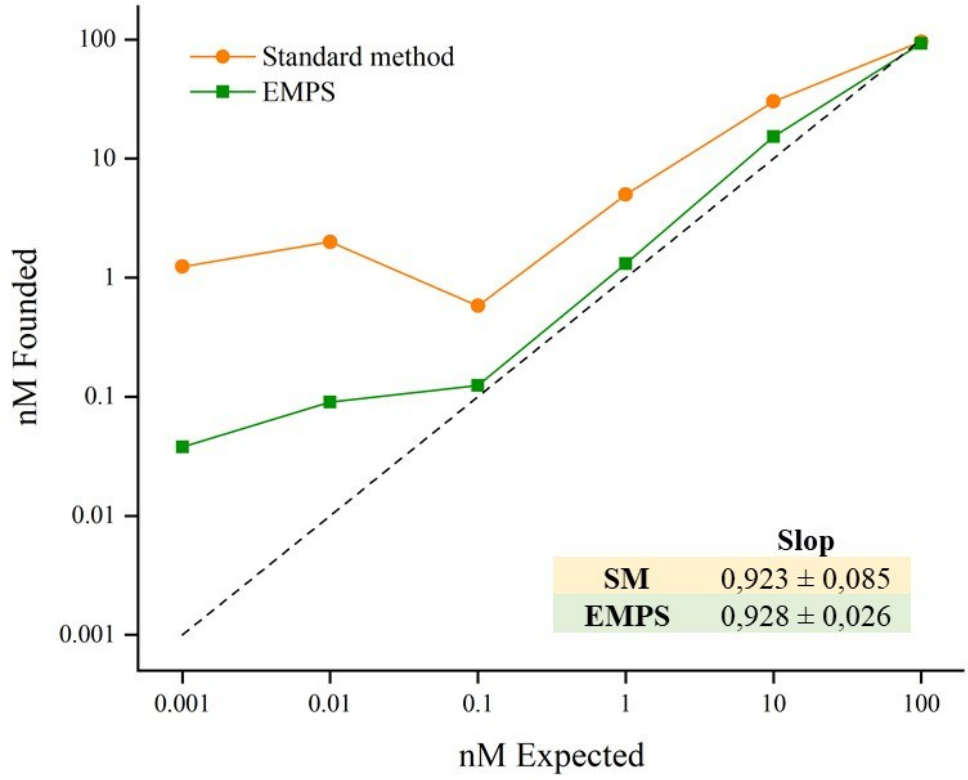

Figure S 5.4: Values calculated of the detected analyte are plotted against the expected values for both methods. The slot is calculated for each curve by Origin software.

6 Materials

Cyclohexane (anhydrous, 99.5%), 1-hexanol (anhydrous, 99%), Triton® X-100, aminopropyl trimethoxysilane [APTMS] (97%), tetraethyl orthosilicate [TEOS] (99.99%), ethyltriethoxysilane (96%)[ETEOS], ammonium hydroxide solution (28% w/v in water, ≥99.99%), 3-(trihydroxysilyl)propyl methylphosphonate monosodium salt (42% w/v in water) [THPMP], fluorescein isothiocyanate isomer I (≥90%) [FITC], sodium phosphate dibasic (>98.5%), sodium phosphate monobasic (>98%), sodium carbonate (≥99.5%), sodium bicarbonate (≥99.5%), methylene blue hydrate, fluoresceinamine isomer I, Atto633 amine, 2-morpholinethanesulfuric acid

monohydrate 99%, glutaraldehyde, N-hydroxysuccinimide [NHS], 1-ethyl-3-(3-dimethylaminopropyl)carbodiamide hydrochloride [EDC], sodium hydroxide Bioextra ≥98%, sodium chloride, hydrochloridric acid Bioreagent for molecular biology, syligad® 184, streptavidin from Streptomyces avidinii ≥ 13 U/mg protein, anti-peroxidase antibody produced in rabbit, agarose molecular biology grade, sodium sulfate, absolute ethanol, bovine serum albumin ≥98%, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfuric acid) diammonium salt, ≥98% HPLC was purchased from Sigma Aldrich (Milan, Italy). PS-MAG-COOH [MNP-COOH] d 270 nm were purchased from MicroParticles GmbH (Berlin, Germany). fluidMAG-ARA, d 100 nm [MNP-COOH] were purchased from Chemicell (Berlin, Germany). Carbon films on 200-mesh copper coated ultra-thin TEM grids was purchased from Electron Microscopy Science (Paris, France). Biotinylated peroxidase was purchased from Invitrogen. Coomassie (Bradford) protein assay Kit was purchased from Thermo Fisher.

7 Methods

Sandwich assay:

General protocol for tracking analysis (high concentrations):

Ab-flu-MNPs (10 µg/mL) were added to a solution of HRP-Bio (5-0.1 µM) in a 1 mL centrifuge tube. The mixture was incubated at 37°C under shaking at 500 rpm, with 10 mM PBS, pH 7.4, used as a buffer. After 2 hours, MNPs were isolated by applying a magnet. The pellet was redispersed in blocking buffer (1% BSA solution in 10 mM PBS, pH 7.4) and incubated for 30 minutes. After magnetic isolation of the pellet, HRP-Ab-MNPs were resuspended in fresh buffer, and St-G-MB-SiNPs were added (500 µg/mL). The mixture was incubated at 37°C with shaking (500 rpm). After 30 minutes, MNPs were isolated from excess St-G-MB-SiNPs by applying a magnet. The pellet was subsequently resuspended in buffer suitable for electrophoresis (1.67 mM $NaSO_4$, 1 mM NaOH and 0.001% agarose; B-1). Next, 10 µL of this suspension was added to the PDMS chamber (100 µL Vol_{tot}). The movement of the particles in the presence of the magnetic field (B = 500 mT) and electric field (E = 1 V, 1 mA) was tracked by measuring the absorbance in each of the three sections into which the chamber was divided at 475 nm and 700 nm for Ab-Flu-MNPs and St-G-MB-SiNPs, respectively. The absorbance was measured every minute for 20 minutes. Next, 5 µL of the visible

isolated fractions were withdrawn, washed twice by two centrifugation/redispersion cycles to remove the agarose fibers and then placed on a grid for TEM analysis.

General protocol for sensitivity (low concentrations): Ab-Atto-MNPs (µg/mL of particles corresponding to 200 nM of Ab) were added to a solution of HRP-Bio at a known concentration (100-0 nM) in a 500 µL centrifuge tube. The mixture was incubated at 37°C with shaking at 500 rpm, and 10 mM PBS, pH 7.4, was used as a buffer. After 2 hours, MNPs were isolated by applying a magnet, followed by removal of 490 µL of supernatant. The pellet was redispersed in blocking buffer (1% BSA solution in 10 mM PBS, pH 7.4) and incubated for 30 minutes. After magnetic isolation of the pellet, HRP-Ab-MNPs were resuspended in fresh buffer, and St-G-FITC-SiNPs were added (µg/mL of particles corresponding to 200-500 nM active sites of streptavidin determined by the Bio-FITC assay). The mixture was incubated at 37°C with shaking (500 rpm). After 30 minutes, HRP-Ab-Atto-MNPs were isolated from excess St-G-FITC-SiNPs by applying a magnet, followed by removal of 490 µL of supernatant. The pellet was washed once in 10 mM MES, pH 4, to remove all the excess SiNPs without disturbing the pellet, which was finally resuspended in 10 µL of 10 mM MES, pH 4.

Standard Method (SM): *5* µL of the isolated fraction was spotted on a clean microscope slide. After the drop was allowed to dry, another 5 µL of the isolated fraction was added over the spot and allowed to dry. The dried spot was analyzed by ChemiDoc™ MP (BioRAD) using the FITC filter.

Electro-magnetophoretic separation (EMPS): The 10 µL sample was further purified in the designed PDMS chamber (50 µL Vol_{tot}). The pellet was then resuspended in buffer suitable for electrophoresis (B-1 for Ab-Flu-MNPs and 10 mM MES, pH 4, for Ab-Atto-MNPs). This suspension was added to the PDMS chamber. The sample was further purified by simultaneously applying a magnetic field (B = 270 mT) and an electric field (500 mV, 1 mA). After 20 minutes, 15 µL samples were carefully withdrawn simultaneously at each electrode. The isolated fractions were concentrated by centrifugation (800 rpm, 4 minutes), followed by removal of 5 µL of the supernatant. The remaining 10 µL was vortexed and spotted (5 µL twice) onto a glass microscope slide.

The dried spot was analyzed by ChemiDoc using an FITC filter, ensuring to always use the same exposure time. The images obtained were evaluated by ImageJ Software, and the intensity of the signal was translated into a value. The intensity value calculated for the amount of St-G-FITC-SiNPs used for the assay is considered 100% of the total amount of streptavidin active sites (SAS) available. Using this value and the intensity value determined for the fraction isolated by standard method and the fraction isolated at the negative electrode by EMPS, the amount of SAS in the isolated fractions was calculated. Streptavidin has 4 binding sites for biotin; however, due to steric hindrance, it is likely that not all of them are effectively available to interact with the biotin-HRP conjugate. Assuming that ¾ of this SAS can interact with the analyte, we correlated the intensity signal measured for the isolated fraction with the amount of analyte isolated.

This experiment was performed several times using independent samples synthesized and characterized each time. The data obtained for each concentration of analyte were evaluated together. The final data were reported as an average of 8 experiments ± SD. The values achieved using the standard method were normalized using the value for the noise signal as the average value among those related to the lower concentrations. Indeed, the data obtained for 1, 10 and 100 pM HRP were found to be similar to the signal measured for the blank sample (0 nM HRP-Bio), indicating that they must be considered noise. The data obtained for EMPS were normalized in the same way but considered the data related to the concentrations of 1-10 pM to be noise, similar to the signal measurement for the blank sample. Furthermore, due to the impossibility of physically isolating the two sides of the chamber before withdrawing the fractions and due to the adhesion of particles to the electrode, it is worth considering that not all of the isolated particles can be effectively collected by withdrawing only 15 μL of the total amount of sample in the chamber (50 μL). To address these disadvantages, we multiplied the values achieved using EMPS by a correction factor of 1.6 (5/3) after normalization.

General protocol for reproducibility and selectivity analysis (low concentrations):

The same procedure as described above was followed to evaluate the selectivity and reproducibility of the methods except for the type and amount of analysis. For the selectivity assay, 100 nM Protein A was used in the assay. For the reproducibility assay, 10 and 0.1 nM HRP-Bio were used in the assay. The robustness of the assay was tested in complex medium such as DMEM and FBS. In this case, the solution of analyte (HRP-Bio) was prepared using such solvents rather than PBS. To these solutions at different HRP-Bio concentrations (0-100 nM), Ab-MNPs were added. The assay and isolation approach were continued by following the same protocol.

8 Analytical Techniques

Dynamic Light Scattering (DLS)

Size and zeta potential (ζ) were measured by Zetasizer NanoZS (Malvern), 633 nm HeNe laser, backscatter angle of 173°. NPs both SiNP and MNP were dispersed at a concentration of 20 μg/mL in DI water (if not differently specified) or in buffers at different pH and concentration. Their size and ζ-potential were analysed in a disposable folded capillary cell (DTS1070) at RT (~25°C) using Malvern Zetasizer. Final values are reported as average of three measurements (n = 3) ± SD.

Transmission Electron Microscopy (TEM):

Images were taken on a JEM-1011 (Joel) equipped with ha tungsten filament operating at 80keV. 5μL of NPs in water (20 μg/mL) was added on 'Carbon Films on 200 Mesh Grids Copper' and allowed to evaporate. Using ImageJ software, *c.a.* 100 NPs per image were analysed statistical values for NP diameter.

UV-Vis analysis:

Multiskan GO (Thermo scientific) microplate reader was used for the absorbance measurements and for the tracking analysis. Cary 300 Scan UV-Vis (Varian) spectrophotometer was used for the UV-Vis analysis of the nanomaterials. Measurements were accomplished in water in plastic disposable cuvette.

Microscope analysis:

Revolution XD Spinning Disk Solution (Yokogawa Head on TiE inverted Microscope with Okolab incubation system and Andor camera 897) was used to simultaneously visualise the presence of MNP and SiNP in the isolated fraction spotted on glass microscope slides.

Fluorescence measurements: quantification of isolated fractions analysis was accomplished using ChemiDOC™ MP (BioRAD) was used to image the fractions isolated and spotted on a glass slide. The fluorescence intensity was converted in a numerical value by means of suitable features of ImageJ software. Cary Eclipse (Varian) was used for the fluorescent analysis for nanomaterial characterization. Measurements were accomplished in water in plastic disposable cuvette. Tecan Infinite M200 microplate reader was used for the fluorescence emission measurements for Bio-FITC quantification.

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